Studies on the Bacterial Formation of a Peptide Antibiotic, Colistin

Part III. On the Biosynthetic Pathway of α,γ -Diaminobutyric Acid and Relationship between Colistin Formation and Amino Acids Metabolism in *Bacillus colistinus* KOYAMA

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The biosynthetic pathway of α,γ -diaminobutyric acid, 6 moles of which are involved in the colistin molecule as a main component, was investigated. On the basis of the isotopic results using aspartic acid- $U^{14}C$ as a precursor and also the finding of transaminase activity between α -ketoglutaric acid and α,γ -diaminobutyric acid, though in reverse reaction, α,γ -diaminobutyric acid was proved to be synthesized from aspartic acid via aspartyl-phosphate and aspartic β -semialdehyde. α,γ -Diaminobutyric acid did not inhibit aspartokinase activity of this bacterium, the first enzyme involved in the process of α,γ -diaminobutyric acid synthesis from aspartic acid, while the end product amino acids such as lysine, threonine and methionine showed inhibition for aspartokinase activity.

On the other hand, α,γ -diaminobutyric acid might be rate-limiting factor in colistin formation, because of stimulatory effect of this diamino acid when added to the medium on colistin production. Furthermore, colistin production appeared to be related with the defect of TCA-cycle and further the resultant increase in activities of the key enzymes such as isopropylmalate synthetase, α -acetolactate synthetase and aspartokinase involved in the biosynthetic pathways of valine, leucine and isoleucine, respectively.

α,γ -Diaminobutyric acid was first found as a component of peptide antibiotic belonging to polymixin family.¹⁻³⁾ In the colistin molecule, 6 moles of α,γ -diaminobutyric acid are involved.⁴⁾ Subsequently, it has been reported by many workers that this amino acid is contained in free state in seeds of higher plants such as *Lathyrus*, *Leguminosae* and *Cruciferae*.⁵⁻⁷⁾ Recently, S. N. Nigam and C. Ressler studied on biosynthesis of α,γ -diaminobutyric acid in *Lathyrus sylvestris* W, and

they confirmed that α,γ -diaminobutyric acid was synthesized from both homoserine and aspartic acid.⁸⁾ However, its detailed biosynthetic pathway is not yet clear.

The purpose of the present investigation was to determine biosynthetic pathway of α,γ -diaminobutyric acid in *Bacillus colistinus*. In addition, the relationship between metabolism of amino acids, including α,γ -diaminobutyric acid and colistin production was studied.

1) P. H. Bell *et al.*, *Ann. New York Acad. Sci.*, **51**, 867 (1949).

2) S. F. Howell, *J. Biol. Chem.*, **186**, 863 (1950).

3) W. Hausmann, *ibid.*, **198**, 405 (1952).

4) K. Suzuki, *Tohoku Yakka Daigaku Kiyo*, **4**, 127 (1957).

5) E. A. Bell, *Nature*, **193**, 1078 (1962).

6) L. Fowden and M. Bryant, *Biochem. J.*, **70**, 626 (1958).

7) C. Ressler, *Science*, **134**, 188 (1961).

8) S. N. Nigam and C. Ressler, *Biochem.*, **5**, 3426 (1966).

MATERIALS AND METHODS

Organisms. *Bacillus colistinus* koyama and *Escherichia coli* NIHJ described in the previous paper⁹⁾ were used throughout this study.

Radioactive compounds and chemicals. Aspartic acid- $U-^{14}C$ (143 mCi/mM) and glutamic acid- $U-^{14}C$ (66 mCi/mM) were purchased from Daiichi Pure Chemical Co. Ltd. Acetyl CoA and acetyl CoA- $l-^{14}C$ which were synthesized by Mr. Sai were kindly supplied for the present studies. α, γ -Diaminobutyric acid, pyridoxalphosphate, N^{α} -acetylaspatic acid, α -ketoisovaleric acid, phosphoenolpyruvate, malic dehydrogenase, ATP, ADP, NAD, NADP were purchased from Sigma Chemical Comp. Other chemicals were purchased from the market.

Condition of incubation of cell suspension. Media and cultural conditions were employed as described in the previous paper.⁹⁾ Cells grown in a synthetic medium for 20 hr on a rotary shaker were harvested at 7,000 rpm for 20 min, washed twice with saline and suspended in M/10 phosphate buffer (pH 7.2). The cell suspension (corresponding to 15 mg dried cell/ml) was used in the following isotopic experiments. Reaction mixture, total volume 20 ml, contained 5 ml of casein hydrolysate (5 mg N/ml), 0.5 ml of 1 M glucose, 10 ml of cell suspension, 0.2 ml of radioactive compounds (5 μ Ci/0.2 ml) and 4.3 ml of M/10 phosphate buffer (pH 7.2). Incubation was carried out at 30°C for 3 hr.

Purification of radioactive colistin. After incubation, reaction mixture with radioactive aspartic acid- $U-^{14}C$ was immediately centrifuged at 12,000 rpm for 20 min. Supernatant was passed through Amberlite IRC 50 (H^+) to remove unincorporated radioactive compounds. The column was completely washed with deionized water and subsequently eluted with 0.5 N HCl. Radioactive fraction, showing one peak in the elution pattern as illustrated in Fig. 1, had biological activity as colistin and this was further confirmed by paper chromatography. The radioactive spot of the fraction had R_f value identical with that of the authentic colistin on paper chromatogram using buthanol-acetic acid-pyridine-water (15:3:10:12 by vol.) as a solvent system.

Isolation and identification of radioactive α, γ -diaminobutyric acid. Radioactive colistin isolated from the

FIG. 1. Elution of Radioactive Colistin from IRC-50 Column.

Supernatant of reaction mixture was applied to the column, which was treated with 15 ml of water, and successively eluted with 0.5 N HCl. The flow rate was approximately 5 ml per hour. Each fraction was 1 ml.

○—○ Colistin activity
▲---▲ Radioactivity

above spot was hydrolyzed with 6 N HCl in a sealed tube for 8 hr by autoclaving. Hydrolyzate was evaporated to dryness. Evaporation was repeated with addition of a small amount of water. The preparation was again dissolved in a small amount of water and was used for paper chromatography and radioautography. Isolation was carried out by paper chromatography using solvent system of buthanol-acetic acid-water (3:1:1 by vol.) and identification by radioautography on Fuji medical X-ray film.

Enzyme assay. After washed cells were disintegrated with sonic oscillator at 10 kC for 15 min, disrupted cells were centrifuged at 12,000 rpm for 30 min. The supernatant was dialyzed and used as crude enzyme preparation. In the case of assay of aspartokinase and N^{α} -acetylaspatic kinase, the fractions precipitated with 0.6 or 0.9 saturated ammonium sulfate were used, respectively. Assay of aspartokinase and N^{α} -acetylaspatic kinase was carried out by Black and Wright's method.¹⁰⁾ Transaminase between α -ketoglutaric acid and α, γ -diaminobutyric acid was investigated by Souda's modified method.¹¹⁾ The assay of transaminase activity between α -ketoglutaric acid

10) Black and Wright, "Methods in Enzymology," V, 821P (1962).

11) K. Souda, *Agr. Biol. Chem.*, **25**, 811 (1961).

9) M. Ito *et al.*, *Agr. Biol. Chem.*, **33**, 262 (1969).

and lysine was done by the similar method. Citrate condensing enzyme was measured by method of S. Ochoa,¹²⁾ isocitrate dehydrogenase by the increase of TPNH at 340 m μ ,¹³⁾ phosphoenolpyruvate carboxylase and pyruvate carboxylase by method of M. Runz Amil,¹⁴⁾ and α -acetolactate synthetase by method of W. Westerfeld.¹⁵⁾ Assay of α -isopropylmalate synthetase was done according to Sai's¹⁶⁾ method of citramalate synthetase, using acetyl CoA and α -ketoisovalerate, instead of pyruvate, as substrates.

Determination of protein. The method of Lowry *et al.* using the Folin-Ciocalteu reagent¹⁷⁾ was employed for this purpose.

Determination of radioactivity. Radioactivity was counted with a Aloka Model PC-10D windowless gas-flow counter.

RESULTS

- 1) *Biosynthetic pathway of α , γ -diaminobutyric acid*
Aspartic acid-U-¹⁴C and glutamic acid-U-¹⁴C were employed as a possible precursor in

TABLE 1. INCORPORATION OF LABELED AMINO ACIDS INTO α , γ -DIAMINO BUTYRIC ACID OF COLISTIN

Labeled amino acid	Colistin activity (unit)	Total count (cpm)	Specific activity (cpm/u)
L-Glutamic acid-U- ¹⁴ C	11,000	815	0.074
L-Aspartic acid-U- ¹⁴ C	11,500	114,000	9.87
L-Aspartic acid-U- ¹⁴ C + α , γ -diaminobutyric acid- ¹² C	13,000	6,980	0.54

Reaction mixture; cell suspension 10 ml (corresponding to 150 mg dried cell), 1 M glucose 0.5 ml, amino acids mixture 5 ml, M/10 phosphate buffer (pH 7.2) Asp-U-¹⁴C 5 μ Ci or Glu-U-¹⁴C 5.7 μ Ci, Total volume 20 ml.

Incubation: 30°C, 3 hr.

12) S. Ochoa, "Methods in Enzymology," I, 685p (1955).

13) N. O. Kaplan, "Methods in Enzymology," III, 892 (1957).

14) M. Runz Amil, *J. Biol. Chem.*, **240**, 3485 (1965).

15) W. W. Westerfeld, *J. Biol. Chem.*, **161**, 495 (1945).

16) T. Sai *et al.*, *Agr. Biol. Chem.*, **32**, 1398 (1968).

17) Lowry *et al.*, *J. Biol. Chem.*, **193**, 265 (1951).

the biosynthesis of α , γ -diaminobutyric acid and incorporation of these labeled amino acids into colistin was examined with cell suspension. As shown in Table I, only very small amount of glutamic acid-U-¹⁴C was incorporated into colistin, while aspartic acid-U-¹⁴C was highly incorporated into colistin. Furthermore, addition of α , γ -diaminobutyric acid-¹²C to cell suspension in the presence of aspartic acid-U-¹⁴C strongly inhibited the incorporation of aspartic acid-U-¹⁴C into colistin, and specific activity of colistin formed in this reaction system was lower than that when aspartic acid-U-¹⁴C alone was supplied. The specific activity of colistin was expressed as radioactivity (cpm) to biological activity (u) shown by colistin. Therefore, aspartic acid but not glutamic acid may be the precursor of α , γ -diaminobutyric acid involved in colistin molecule.

To confirm the above incorporation test, labeled colistin was isolated from the supernatant of the incubated mixture with cell suspension as described in the Methods, and, adding 10 mg of the authentic colistin as

FIG. 2. Radioautogram of Hydrolysate of Labeled Colistin Formed from Aspartic Acid-U-¹⁴C (left) and Ninhydrin Spots (right).

carrier, it was hydrolyzed with 5N HCl. After hydrolyzation, the labeled preparation was subjected to a chromatography, in which solvent system used was buthanol-acetic acid water (3:1:1 by vol.). The paper developed was completely dried and radioautography was carried out. Fuji medical X-ray film was exposed with the paper developed for 10 days, after which the paper separated from X-ray film was colored with ninhydrin for the detection of amino acids. Figure 2 shows paperchromatogram and radioautogram of hydrolyzate of labeled colistin formed in the presence of aspartic acid- $U-^{14}C$ with cell suspension. Radioactive spots were found in the sites corresponding to authentic α, γ -diamino-

butyric acid and threonine, although radioactive spot of threonine was far weaker than that of α, γ -diaminobutyric acid.

Furthermore, to clarify the intensity distribution in radioactivity of the components of labeled colistin, each spot on the paper chromatogram colored with ninhydrin and bromcresolgreen was cut off, eluted with water and a aliquot of eluates was counted with gas-flow counter. The result of intensity distribution of radioactive compounds obtained is shown in Table II. Most of radioactivity of the components of colistin molecule was found in α, γ -diaminobutyric acid, which occupied 83%, while radioactivity in threonine was distributed to an extent of 17%. Thus, it was confirmed that α, γ -diaminobutyric acid was derived from aspartic acid in the present bacterium in the same manner as was found in *Lathyrus sylvestris* W.

TABLE II. DISTRIBUTION OF RADIOACTIVITY IN THE CASE OF INCORPORATION OF L-ASPARTIC ACID- $U-^{14}C$ INTO COLISTIN

Component of colistin	Total count (cpm)	Distribution (%)
Leucine	0	0
Threonine	210	17
α, γ -Diaminobutyric acid	1160	83
6-Methyloctanoic acid and isooctanoic acid	0	0

Hydrolysate of labeled colistin was carried out paper chromatography with BuOH:AcH:H₂O (3:1:1 by vol.).

Radioactivity was measured with gas-flow counter. Radioactivity of fatty acids was measured with liquids scintillation counter.

From these results, two biosynthetic pathways as illustrated in Fig. 3 were assumed to be possible. One of them is the conversion of aspartate to α, γ -diaminobutyric acid via β -aspartylphosphate and aspartate- β -semialdehyde and another is that α, γ -diaminobutyric acid is synthesized from aspartic acid via the corresponding N-acetylated intermediates, such as N-acetylaspargate, N-acetyl- γ -aspartylphosphate, N-acetylspartic- γ -semialdehyde and N-acetyl- γ -aminobutyric acid. The formation

FIG. 3. Probable Biosynthetic Pathways of α, γ -Diaminobutyric Acid.

of α, γ -diaminobutyric acid from N-acetyl- γ -aminobutyric acid may be catalyzed by a hydrolytic enzyme like acetylorinithinase of *E. coli*.¹⁸⁾

Then, enzyme activities related to the first step, i.e. those of aspartokinase and N $^{\alpha}$ -acetyl-aspartokinase activities were investigated, using the enzyme preparation precipitated with 0.6 saturated $(\text{NH}_4)_2\text{SO}_4$. As shown in Table III, aspartokinase activity was 0.374,

TABLE III. ASPARTOKINASE AND N $^{\alpha}$ -ACETYL-ASPARTOKINASE ACTIVITIES

Substrate	$-\log T$ (at 540 m μ)
L-Aspartic acid	0.374
N $^{\alpha}$ -Acetylaspatic acid	0.012
N $^{\alpha}$ -Acetylaspatic acid*	0.002

The assay mixture contained ATP 10 μ moles, $\text{NH}_2\text{OH}\cdot\text{HCl}$ 800 μ moles, MgCl_2 10 μ moles, L-Asp. 100 μ moles, crude enzyme preparation (fraction precipitated with 0.6 saturated $(\text{NH}_4)_2\text{SO}_4$, 7.2 mg protein per ml) Incubation; at 26°C, for 30 min.

After addition of FeCl_3 reagent, the optical density of the aspartohydroxamate-ion complex was measured at 540 m μ .

* Fraction precipitated with 0.9 saturated $(\text{NH}_4)_2\text{SO}_4$ was used as enzyme solution, and the amount of enzyme protein was the same as preliminary experiment.

while N $^{\alpha}$ -acetylasparkokinase activity was very weak and only 0.012 with the reaction mixtures containing 7.2 mg of crude enzyme protein in each tube. Furthermore, when N $^{\alpha}$ -acetylasparkokinase activity was assayed with the preparation obtained with 0.9 saturated ammonium sulfate, the activity was also as weak as the above enzyme preparation. Thus, the enzyme preparations so far tested were assumed to exhibit aspartokinase alone and accordingly the alternative scheme II was ruled out from the assumption.

Biosynthesis of α, γ -diaminobutyric acid from aspartic acid appears to involve a conversion of α, γ -diaminobutyric acid to aspartic β -semialdehyde which is the reverse reaction of the

final step in this biosynthesis. As to the transamination of β -semialdehyde compounds, Fincham¹⁹⁾ once observed that mycelial extracts of *Neurospora crassa* catalyze transamination of ornithine to α -ketoglutaric acid to form glutamic semialdehyde and glutamic acid, respectively. Then, transamination between α, γ -diaminobutyric acid and several kinds of α -keto acids was preliminarily tested as using the crude enzyme extracts, where transamination of α, γ -diaminobutyric acid was predicted to result in the formation of aspartic semialdehyde. Of α -keto acids tested, only α -ketoglutaric acid gave positive result in the formation of glutamic acid and thus this α -keto acid seemed to be an active substrate for the transamination, whereas pyruvic acid or oxaloacetic acid, reacting with α, γ -diaminobutyric acid, was not converted into the corresponding amino acids such as alanine or aspartic acid. Therefore, α, γ -diaminobutyric acid and α -ketoglutaric acid were used as substrates in the reaction system of transamination, in which glutamic acid formed was quantitatively assayed according to Giri's method.²⁰⁾

Table IV shows the formation of glutamic acid resulting from the transamination of α, γ -diaminobutyric acid to α -ketoglutaric acid.

TABLE IV. TRANSAMINATION BETWEEN L- α, γ -DIAMINOBTYRIC ACID AND α -KETOGLUTARIC ACID

Reaction system	Activity*
Complete system	1.59
Minus L- α, γ -diaminobutyric acid	0.080
Minus α -ketoglutaric acid	0.073
Minus enzyme	0.073

Incubation mixture contained α, γ -diaminobutyric acid 20 μ moles, α -ketoglutaric acid 20 μ moles, pyridoxal phosphate 1 μ mole, crude enzyme (3.5 mg protein), phosphate buffer 100 μ moles (pH 8.0). Incubation carried out at 37°C, for 60 min.

* Glutamate formed (μ moles)/mg of protein/60 min.

- 19) H. R. S. Fincham, *Biochem. J.*, **53**, 313 (1953).
20) E. V. Giri, *Nature*, **170**, 1025 (1952).

18) Henry J. Vogel, *J. Biol. Chem.*, **218**, 97 (1956).

Glutamic acid there formed was well determined only in the complete system but not, if any, in reaction systems lacking either one of amino donor, amino acceptor or crude enzyme solution. Thus, the transamination between α, γ -diaminobutyric acid and α -ketoglutaric acid, though reversedly to the final step in the formation process of α, γ -diaminobutyric acid, was well performed with the crude enzyme extracts of this organism. In this case, in order to obtain active enzyme extracts a special caution was required at the disruption of cells with sonic oscillator, where $1 \mu\text{mole}$ of pyridoxal-5'-phosphate and $1 \mu\text{mole}$ of glutathione were added to 1.0 ml of the cell suspension. If cells were disrupted in the absence of pyridoxal-5'-phosphate, this transamination activity was not at all detectable in the resultant cell extracts, so that pyridoxal-5'-phosphate seemed to act as a protecting agent for the enzyme activity.

Furthermore, identification of reaction products were carried out with paperchromatography using butanol-acetic acid-water (4:1:2

by volume) as a solvent system. As shown in Fig. 4, a spot corresponding to glutamic acid as sprayed with 0.5% ninhydrin solution was identified by comparing it with that of the authentic compound. On the other hand, when the developed paper was sprayed with ammoniacal silver nitrate solution, a spot of the other reaction product, aspartic β -semialdehyde, was though not distinctly separable from that of α -ketoglutaric acid because of tailing, well-discernible when compared with the spot of the authentic compound.

From the results, it is concluded that α, γ -diaminobutyric acid is synthesized from aspartic acid via aspartylphosphate and aspartic β -semialdehyde which in turn is successively transaminated with glutamic acid as illustrated in Fig. 3-I, and that no N-acetylation of aspartic acid participates in the reaction series of this biosynthesis.

2) Amino acid metabolism and colistin formation

As reported previously,⁹⁾ valine, leucine and isoleucine are abundantly but glutamic acid is poorly pooled within the colistin-producing cell, whereas the reverse is the case within the colistin-not-producing cell. The former cell is obtained from the culture in the synthetic medium containing starch as carbon source while the latter from the culture in the glucose-bouillon medium. The above fact suggests that in colistin producing cells, the metabolism of amino acids is so altered as to produce much more of the amino acids of pyruvate series such as valine and leucine as well as of aspartate series such as isoleucine, including α, γ -diaminobutyric acid, a main amino acid component of colistin and is derived from aspartic acid as already mentioned, thus providing components of colistin. Then, activities of key enzymes and related ones in the biosyntheses of these amino acids were investigated, by using enzyme preparations obtained from both cells above mentioned.

As summarized in Table V, the enzyme

FIG. 4. Paper Chromatogram for Identification of Reaction Product.

Solvent system: butanol : acetic acid : water (4:1:2 by vol.).

Left: a spray reagent used 0.5% ninhydrin dissolved in 95% acetone.

Right: ammoniacal silver nitrate was sprayed.

Abbreviation: Glu, glutamic acid; DAB, α, γ -diaminobutyric acid; α -Kg, α -ketoglutaric acid; Asp- β -semialdehyde, Aspartic β -semialdehyde.

TABLE V. COMPARISON OF ENZYME ACTIVITIES BETWEEN COLISTIN PRODUCING CELLS* AND COLISTIN NOT-PRODUCING CELLS**

Enzymes	Colistin producing cells	Colistin not-producing cells	Units of enzymes
Citrate condensing enzyme	0	9.25	$\mu\text{moles/min/mg protein}$
Isocitrate dehydrogenase	0	13.9	"
Phosphoenolpyruvate carboxylase	16.6	1064	cpm/mg protein/15 min
Pyruvate carboxylase	0	0	"
α -Acetolactate synthetase (pH 6.0)	38.8	22.1	$\text{u/mg protein/60 min}$
" (pH 8.0)	15.8	7.5	"
α -Isopropylmalate synthetase	4.9	1.4	$\text{m}\mu\text{moles/mg protein/hr}$
Aspartokinase	0.492	0.133	$-\log T \text{ at } 540 \text{ m}\mu/7.2 \text{ mg protein/hr}$

* Cells grown in synthetic medium, colistin formed 5000~7000 u/ml.

** Cells grown in glucose bouillon medium, colistin formed 0~1000 u/ml.

preparation obtained by fractionation with 0.6 saturated ammonium sulfate from the crude extract of the colistin-producing cell exhibited considerable activities of α -acetolactate synthetase (pH 6.0 and pH 8.0), α -isopropylmalate synthetase and aspartokinase, respectively. Whilst, in this cell, activities of some TCA cycle enzymes such as citrate condensing enzyme and isocitrate dehydrogenase were entirely, if any, eliminated and a little activity of phosphoenolpyruvate carboxylase was detected. In contrast, the reverse was the case in these enzymatic activities of the colistin-not-producing cell and considerable activity was observable in the enzymes involved in TCA cycle, especially a remarkable one in phosphoenolpyruvate carboxylase, while the enzymes concerned with biosyntheses of valine, leucine and isoleucine, such as α -acetolactate synthetase, α -isopropylmalate synthetase and aspartokinase, showed lower activity compared with those of the colistin-producing cells. Both of the cells, in addi-

tion, were defective in pyruvate carboxylase. Thus, the colistin-producing cell is well characterized by the defect of the key enzymes performing in TCA cycle but enrichment of the key enzyme involved in the biosyntheses of valine, leucine and isoleucine. This may give rise to enrichments of these amino acids pooled within the cell, resulting in the formation of colistin.

In view of the distribution of amino acids composed of colistin molecule, it is assumed that an inclination to the formation of α , γ -diaminobutyric acid, exceeding that of other amino acids, especially of threonine, occurs in the colistin-producing cell. Since this diamino acid is derived from aspartic acid which in turn may result from oxaloacetic acid possibly formed by phosphoenolpyruvate carboxylase, though of low activity, aspartokinase is of importance not only for biosyntheses of lysine, threonine and isoleucine, as is well

TABLE VI. EFFECT OF VARIOUS END PRODUCTS ON ASPARTOKINASE ACTIVITY

Additions	$-\log T$ (at 540 m μ)	Inhibition (%)
None	0.495	0
L-Lysine 50 mM	0.125	74.7
L-Lysine 5 mM	0.445	10.1
L-Threonine 50 mM	0.285	42.4
" 5 mM	0.445	10.1
L-Methionine 50 mM	0.382	22.8
" 25 mM	0.482	2.6
L- α , γ -Diaminobutyric acid 50 mM (DAB)	0.550	0
L- α -Aminobutyric acid 50 mM	0.550	0
L-Lysine + L-DAB 50 mM each	0.140	71.7
L-Threonine + L-DAB 50 mM each	0.255	48.4

The assay mixture contained ATP 10 μmoles , $\text{NH}_2\text{OH}\cdot\text{HCl}$ 800 μmoles , MgCl_2 10 μmoles , L-Asp. 100 μmoles , each end product inhibitor and enzyme solution (8.6 mg of protein). Total volume was 1.0 ml and incubation was carried out at 26°C, for 30 min. After addition of FeCl_3 reagent, the optical density of the aspartohydroxamate-ion complex was measured at 540 m μ .

known, but also for the formation of α,γ -diaminobutyric acid. It is also known that activity of aspartokinase is variously governed by the endproduct amino acids and α -aminobutyric acid, but effect of α,γ -diaminobutyric acid on it is as yet unknown. Then, effects of this diamino acid as well as other monoamino acids mentioned above were investigated, using crude enzyme extracts obtained from the colistin-producing cell. The results obtained are shown in Table VI. An inhibition was observed, though to various extents, by addition of L-lysine, L-threonine and L-methionine, respectively, to the reaction mixture, whereas α -aminobutyric acid and α,γ -diaminobutyric acid were of no effect. Thus, inhibition rate showed 74.7% by L-

lysine, 42.4% by L-threonine and 22.8% by L-methionine, where the concentration of each amino acid required for the maximal inhibition under assay conditions were almost the same and 5×10^{-2} M. Furthermore, the concerted inhibitory effect of L-threonine and L-lysine on aspartokinase activity was observed in *Bacillus polymixa* and other bacteria.²¹⁻²⁴ Such a concerted effect was pronounced on the action of aspartokinase of the present organism when both of these amino acids were concomitantly supplied at more diluted concentration of 5.0 mM to the reaction mixture. As shown in Table VII, 5.0 mM of threonine or lysine showed either none or a little inhibitory effect but the coexistence of these amino acids at each concentration of 5.0 mM in the reaction mixture gave rise to a remarkable inhibition and the rate attained to 93.8%, although more diluted concentration such as 0.5 mM of each amino acid brought about no concerted inhibition. In respect of this feature in the concerted inhibition of aspartokinase by lysine and threonine, the present bacterium, *B. colistinus*, well coincides with *B. polymixa*. When α,γ -diaminobutyric acid as well as α -aminobutyric acid were supplied either individually or concomitantly with lysine alone or with lysine and threonine, they should no, if any, effect, as seen from Tables VI and VII, so that the releasing effect of these monoamino and diaminobutyric acids from the inhibition by lysine, either alone or together with threonine, was almost eliminated in this case, contrary to the case of this effect of α -aminobutyric acid on *Bacillus megaterium* L-2 enzyme.²⁵ Such an inertness of α,γ -diaminobutyric acid, including the fact that

TABLE VII. CONCERTED FEEDBACK INHIBITION OF ASPARTOKINASE ACTIVITY

Additions	$-\log T$ (at 540 m μ)	Inhibition (%)
None	570	0
L-Lysine 5 mM	570	0
L-Threonine 5 mM	570	0
L- α,γ -Diaminobutyric acid (DAB) 5 mM	570	0
L-Lysine + L-threonine 5 mM each	35	93.8
L-Lysine + L-threonine + DAB 5 mM each	45	92.1
L-Lysine + L-threonine 2.5 mM each	185	67.5
L-Lysine 2.5 mM + L-threonine 2.0 mM	195	65.7
L-Lysine 2.5 mM + L-threonine 1.5 mM	320	43.8
L-Lysine 2.5 mM + L-threonine 1.0 mM	395	30.7
L-Lysine 2.5 mM + L-threonine 0.5 mM	500	12.2
L-Lysine 2.0 mM + L-threonine 2.5 mM	165	71.0
L-Lysine 1.5 mM + L-threonine 2.5 mM	255	55.2
L-Lysine 1.0 mM + L-threonine 2.5 mM	380	33.3
L-Lysine 0.5 mM + L-threonine 2.5 mM	470	17.5
L-Lysine 0.5 mM + L-threonine 0.5 mM	570	0

The assay methods was same as Table VI. 2.5 mg of crude enzyme protein was used.

21) H. Paulus and E. Gray, *J. Biol. Chem.*, **239**, PC 4008 (1964).

22) P. Datta and H. Gest, *Proc. Natl. Acad. Sci. U. S.*, **52**, 1004 (1964).

23) K. Nakayama *et al.*, *Agr. Biol. Chem.*, **30**, 611 (1966).

24) R. Miyajima *et al.*, *J. Biochem. (Japan)*, **63**, 139 (1968).

25) K. Sasaki *et al.*, *Amino acid and Nucleic acid*, **10**, 60 (1964).

there exists no activating enzyme for this diaminobutyric acid in the present bacterium²⁶⁾ as mentioned previously, may be noticeable. According to E. R. Stadtman's concept,²⁷⁾ aspartokinase of *E. coli* is distinguishable in its isozymes specific for the biosynthesis of lysine or threonine. This may be applicable to the present aspartokinase receiving the concerted inhibition by lysine and threonine, although this synergism may be supposed to be always broken by the mobilization of threonine to the biosynthesis of colistin. Thus, the aspartokinase action may be inclined to the synthesis of α, γ -diaminobutyric acid especially on the basis of the inertness of this diaminobutyric acid and accordingly this may result in more formation of α, γ -diaminobutyric acid than that of threonine to be involved abundantly in colistin molecule. Of the amino acids which are abundantly biosynthesized in the colistin-producing cell, valine and isoleucine may be mobilized to form colistin fatty acids such as 6-methyloctanoic acid and isooctanoic acid,⁹⁾ so that these amino acids, contrary to leucine and threonine, may not be comprized in colistin peptide.

Furthermore, as indicated in Table VIII,

TABLE VIII. EFFECT OF α, γ -DIAMINO BUTYRIC ACID ON COLISTIN FORMATION

α, γ -Diaminobutyric acid (mg/ml)	Colistin formed (u/ml)	Activity ratio (%)
0	800	100
1.0	1100	137
2.5	1100	137
5.0	1300	162
10.0	1800	225

Reaction mixture; cell suspension 2 ml (corresponding to 30 mg dried cell), amino acids mixture 1.0 ml, 1 M glucose 0.1 ml, M/10 phosphate buffer (pH 7.2), α, γ -diaminobutyric acid, total volume 4.0 ml.

Incubation; at 30°C, for 3 hr.

the more concentrated α, γ -diaminobutyric acid was supplied to the culture of heavy inoculum in the medium containing amino acid mixture and glucose, the more colistin was formed during 3 hr incubation. In contrast to the inactivity of the colistin fatty acids such as 6-methyloctanoic acid and isooctanoic acid as mentioned previously,⁹⁾ this may be ascribed, at least partially, to the above inertness of α, γ -diaminobutyric acid which in turn seems likely to be rate-limiting factor for the biosynthesis of colistin.

DISCUSSION

In bacteria, lysine or ornithine is biosynthesized via N-succinylated or N-acetylated intermediates, protecting each α -amino group from other attacks.²⁸⁻³⁰⁾ In contrast, in fungi or yeast such as *Neurospora crassa* or *Torula utilis*, these basic amino acids are biosynthesized without such protection process of α -amino group as N-acetylation. Thus, bacterial biosyntheses of ornithine and lysine, both of which are diamino-*n*-fatty acid of C₅ or C₆, are distinguishable from fungal ones by the protection process of α -amino group. In turn, α, γ -diaminobutyric acid, also diamino-*n*-fatty acid of C₄, was proved to be biosynthesized without any N-acetylation in the present bacterium, as already mentioned. The biosynthetic type in this case is thus assumed rather to belong to the fungal type. The abnormality of α, γ -diaminobutyric acid in its biosynthetic type may be based upon either the kind of bacteria or the difference in length of carbon chain of those diamino fatty acids. In any case, it is noticeable to find non-N-acetylated biosynthesis of diamino fatty acid in bacteria.

In the present study, the deaminated pro-

26) M. Ito, K. Aida and T. Uemura, Abstracts of papers, the 41th General Meeting of Japanese Biochemical Society, Tokyo, Japan, September, 1968 p. 535.

27) E. R. Stadmen, *Adv. in Enzymology*, **28**, 65 (1966).

28) H. J. Vogel., *Proc. Natl. Acad. Sci. U. S.*, **39**, 578 (1953).

29) C. Gilvarg, *Biochem. Biophys. Acta*, **24**, 216 (1957).

30) C. Gilvarg, *J. Biol. Chem.*, **234**, 2955 (1959).

duct of α, γ -diaminobutyric acid in the transamination with α -ketoglutaric acid was proved to be aspartic β -semialdehyde by the comparison with the spot of the authentic compound on the paperchromatogram. It is yet doubtful whether the deaminated product is aspartic β -semialdehyde alone or contaminated with α -keto- γ -aminobutyric acid. Further studies to confirm the deaminated product are now in progress.

Since the formation of this antibiotic was well stimulated by addition of α, γ -diaminobutyric acid to the culture medium and colistin fatty acids were of no effect as previously mentioned, α, γ -diaminobutyric acid is considered to be rate-limiting factor in colistin formation. In this connection, glutamic acid which is assumed as a main amino donor to aspartic β -semialdehyde in the transamination is of importance in the formation of α, γ -diaminobutyric acid. As the colistin-producing cell of this bacterium is defective in TCA cycle, glutamic acid is considered to be formed via

other pathway than TCA cycle like in *Gluconobacter suboxydans* which also lacks TCA cycle.³¹⁾ This will be further inquired in future.

It is noticeable that the colistin-producing cell is defective in TCA cycle, thus resulting in the inclination to an abundant formation of valine and leucine in amino acid metabolism. In this respect, the formation of peptide antibiotic, colistin, is assumed as a kind of fermentation and in place of cell protein which requires for its synthesis large amount of ATP yielded through TCA cycle, this peptide may be formed due to the defect of TCA cycle in the colistin-producing cell. Since the present organism lacks α, γ -diaminobutyryl-s-RNA synthetase,²⁶⁾ the colistin formation, of which mechanism, however, is as yet obscure, may be dispensable for rich ATP because of its deviation from the ribosomal peptide biosynthesis.

31) Vernon H. Cheldelin, *Metabolic Pathways in Microorganisms*, John Wiley & Sons, Inc. New York (1960).